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F-T Jet Fuel Reverse Mutation Assay and Chromosome Aberration Test

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14. ABSTRACT
Two assays were performed in order to investigate the potential of Fischer Tropsch (F-T) jet fuel for its ability to induce genotoxicity. First, to determine its ability to induce gene mutations, the plate incorporation test was performed with the Salmonella typhimurium strains TA 98, TA 100, TA 102, TA 1535 and TA 1537. No toxic effects of the test item were noted in any of the five tester strains used up to the highest dose group evaluated (with and without metabolic activation). F-T jet fuel did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used. Therefore, F -T jet fuel is considered to be non-mutagenic in this bacterial reverse mutation assay. Second, to investigate a possible potential of F -T jet fuel for its ability to induce structural chromosome aberrations in human lymphocytes in vitro, a chromosome aberration assay was carried out. During the described in vitro chromosomal aberration test and under the experimental conditions reported, the test item F-T jet fuel did not induce structural chromosomal aberrations in human lymphocyte cells. Therefore, F-T jet fuel is considered to be non-clastogenic in this chromosome aberration test.

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PREFACE

This study was conducted to comply with: Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended and promulgated on June 20, 2002 (BGB l. I NrAO S. 2090), revised October 31, 2006 (BGB 1. I Nr. 50 S.2407). The study also complied with the Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (1998).

This study was assessed in compliance with the project protocol, the study plan and the Standard Operating Procedures of BSL BIOSERVICE. The study and test facility were periodically inspected by the Quality Assurance. These inspections and audits were carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. The final report of the study was audited. There were no circumstances that may have affected the quality or integrity of the study.

All animal procedures used in this study were in strict accordance with the European Community Council Directive of 24 November 1986 (86-609/EEC) (protection of animals used for experimental and other scientific purposes) and Decree of 20 October 1987 (87-848/EEC).

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1.0 EXECUTIVE SUMMARY

In order to investigate the potential of F-T jet fuel for its ability to induce genotoxicity, two assays were performed. First, to determine its ability to induce gene mutations, the plate incorporation test was performed with the *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537. In two independent experiments, several concentrations of the test item were used. Each assay was conducted with and without metabolic activation. The concentrations, including the controls, were tested in triplicate. The following concentrations of the test item were prepared and used in the experiments: Experiment I: 0.0316, 0.100, 0.316, 1.0, 2.5 and 5.0 µL/plate; and Experiment II: 0.1875, 0.375, 0.75, 1.5, 3.0 and 5.0 µL/plate. No precipitation of the test item on the agar plates was observed in any of the five tester strains used in Experiment I and II (with and without metabolic activation). However, a clouding of the S9 mix and the S9 substitution buffer, respectively, after addition of the test item solution was noted at a concentration of 0.316 µL/plate and higher (with and without metabolic activation) in Experiment I and at a concentration of 0.375 µL/plate and higher (with and without metabolic activation) in Experiment II. No toxic effects of the test item were noted in any of the five tester strains used up to the highest dose group evaluated (with and without metabolic activation) in Experiment I and II. No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with F-T jet fuel at any concentration level, neither in the presence nor absence of metabolic activation in Experiment I and II. The reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiments. In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, F-T jet fuel did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used. Therefore, F -T jet fuel is considered to be non-mutagenic in this bacterial reverse mutation assay.

Second, to investigate a possible potential of F -T jet fuel for its ability to induce structural chromosome aberrations in human lymphocytes *in vitro* a chromosome aberration assay was carried out. The chromosomes were prepared 24 hours after start of treatment with the test item. The treatment interval was 4 hours with and without metabolic activation (Experiment III) and 4 hours with and 24 hours without metabolic activation (Experiment IV). Two parallel cultures were set up. Per culture 100 metaphases were scored for structural chromosomal aberrations. The following concentrations were evaluated. For Experiment III with metabolic activation (4 hours treatment, 24 hours preparation interval), 1.0, 2.5 and 5 µL/mL were used. Without metabolic activation (4 hours treatment, 24 hours preparation interval), 0.16, 0.50, 1.58 and 5 µL/mL were used. For Experiment IV with metabolic activation (4 hours treatment, 24 hours preparation interval), 3, 4 and 5 µL/mL were used. Without metabolic activation (24 hours treatment, 24 hours preparation interval), 0.50, 1.58 and 5 µL/mL were used. A reduction of the mitotic index was observed in all experiments without metabolic activation. In the experiments with metabolic activation, no reduction of the mitotic index was found. In Experiment III and IV no biologically relevant increase of the aberration rates was noted after treatment with the test item with and without metabolic activation. The aberration rates of all dose groups treated with the test item were within the historical control data of the negative control. No substantial increase in the frequencies of polyploid metaphases was found after treatment with the test item compared to the frequencies of the controls. EMS (ethylmethanesulfonate, 400 and 600 µg/mL) and CPA (cyclophosphamide, 7.5 µg/mL) were used as positive controls. They showed a

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distinct and biologically relevant increase of cells with structural chromosome aberrations above our historical control level. In conclusion, it can be stated that during the described *in vitro* chromosomal aberration test and under the experimental conditions reported, the test item F-T jet fuel did not induce structural chromosomal aberrations in human lymphocyte cells. Therefore, F-T jet fuel is considered to be non-clastogenic in this chromosome aberration test.

2.0 INTRODUCTION

The U.S. Air Force is developing alternative fuels with the aim of decreasing dependence on foreign oil. All new fuels are potentially hazardous to Air Force personnel and require evaluation. Fischer Tropsch (F-T) fuel, the first alternative jet fuel to be certified for use in the U.S. Air Force fleet, is undergoing toxicological evaluation by the 711 Human Performance Wing, Human Effectiveness Directorate, Biosciences and Performance Division, Applied Biotechnology Branch (711 HPW/RHPB). These microbial mutagenicity and chromosome aberration assays are part of this evaluation.

Microbial mutagenicity assays can rapidly detect mutagenic activity in a wide range of chemical classes. Genotoxic evaluations of chemicals utilizing microbial mutagenicity assays are short term, sensitive, and reliable tests performed *in vitro* for assessing mutagenic potential (Mortelmans and Zeiger, 2000). Many chemicals that result in mutagenic responses in the *Salmonella* assay have been found to be potentially mutagenic and carcinogenic to laboratory animals and humans (Zeiger, 1998).

Bacterial reverse mutation assays use amino acid requiring strains of *Salmonella typhimurium* to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs. The principle of these bacterial reversion assays is that they detect mutations which functionally reverse mutations present in the tester strains and restore the capability to synthesize an essential amino acid (Ames *et al.*, 1973; Claxton *et al.*, 1987; Maron and Ames, 1983). The purpose of this study is to establish the potential of the test item to induce gene mutations in bacteria by means of two independent *S. typhimurium* reverse mutation assays.

The *S. typhimurium* histidine (his) reversion system measures his -·to his + reversions. The *S. typhimurium* strains are constructed to differentiate between base pair (TA 100, TA 1535 and TA 102) and frameshift (TA 98 and TA 1537) mutations (Maron and Ames, 1983). These assays directly measure heritable DNA mutations of a type which is associated with adverse effects (McCann *et al.*, 1975; McCann and Ames, 1976; Zeiger *et al.*, 1988; 1992). Point mutations are the cause of many human genetic diseases and there is substantial evidence that somatic cell point mutations in oncogens and tumor suppressor genes are involved in cancer in humans and experimental systems (Ames *et al.*, 1977).

The tester strains have several features that make them more sensitive for the detection of mutations. The specificity of the strains can provide useful information on the types of mutations that are induced by mutagenic agents. According to the direct plate incorporation method, the bacteria are exposed to the test item with and without metabolic activation and plated on selective medium. After a suitable period of incubation, revertant colonies are counted (Maron and Ames, 1983). At least five different concentrations of the test item are tested with

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approximately half log (i.e., $\sqrt{10}$) intervals between test points for an initial test. More narrow spacing between dose levels may be appropriate when a dose response is investigated. For soluble, non-toxic test compounds, the recommended maximum test concentration is 5 mg/plate or 5 µL/plate. To validate the test, reference mutagens are tested in parallel to the test item (Gatehouse *et al.*, 1994).

The purpose of the *in vitro* chromosome aberration (CA) test is to identify agents that cause structural chromosome aberrations in stimulated cultured human lymphocytes. The chromosomes are prepared 24 hours after start of treatment with the test item. The treatment interval is 4 hours with and without metabolic activation (Experiment III) and 4 hours with and 24 hours without metabolic activation (Experiment IV). Two parallel cultures are set up. Per culture, 100 metaphases are scored for structural chromosomal aberrations.

Chromosome aberration assays aim to detect the induction of chromosome breakage (clastogenesis). Although substances produce structural chromosome aberrations by a variety of mechanisms, the endpoint is a discontinuity in the chromosomal DNA which is left unrejoined, or rejoined inaccurately to produce a mutated chromosome. Many of these changes will be lethal to the cell during the first few cell cycles after their induction, but are used as indicators of the presence of non-lethal changes such as reciprocal translocations, inversions and small deletions. These more subtle changes may have important consequences in both germ and somatic cells. Chromosomal mutations and related events are the cause of many human genetic diseases and there is substantial evidence that these changes including oncogens and tumor suppressor genes are involved in cancer in humans and experimental systems. CAs are generally evaluated in first post-treatment mitoses. The majority of chemical mutagens induce aberration of the chromatid type, but chromosome type aberrations also occur.

Short-term cultures of peripheral blood lymphocytes are stimulated to divide by the addition of a mitogen (e.g., phytohemagglutinin: PHA) to the culture medium. Mitotic activity begins at about 40 hours after PHA stimulation and reaches a maximum at around 3 days. The chromosome constitution remains diploid during short-term culture. Treatments should commence at around 48 hours after culture initiation, when the cells are actively proliferating and should be sampled first at about 24 hours later (1 - 1.5 fold of the normal cell cycle time), i.e., at 72 hours after culture initiation (the cycle time of lymphocytes, except first cycle averages about 11 - 17 hours). The cell cycle of the actual lymphocyte cultures is monitored using a BrdU (bromodeoxyuridine)-labeling technique. If toxicity occurs or cell cycle delay is indicated, an additional sampling time should be used at about 24 hours after the first fixation (e.g., 48 hours after beginning of treatment or 96 hours after culture initiation).

At least three concentrations of the test item should be used at fixation time (24 hours). The highest concentration should be in the toxic range and should show a significant reduction in mitotic index or in degree of cell confluency (50 percent or greater). The lowest dose should be in the range of the negative control. In the additional sampling time (delayed fixation time = 48 hours) during the second experiment, the same dose that induced a suitable degree of mitotic inhibition at the earlier fixation time should be chosen. Though the purpose of the assay is to detect structural chromosome aberrations, it is important to report polyploidy and/or

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endoreduplication when this is seen. To validate the test, reference mutagens are tested in parallel to the test item.

3.0 MATERIALS AND METHODS

3.1 Characterization of the Test Substance

F-T jet fuel, Synthetic Jet Fuel (Batch No.: POSF5109), was provided by Air Force Research Laboratory, Propulsion Directorate, Fuels Branch (AFRL/RZPF). The purity of the test substance was 100 percent. Routine hygienic procedures were sufficient to assure personnel health and safety.

3.2 Mammalian Microsomal Fraction S9 Mix

An advantage of using *in vitro* cell cultures is the accurate control of the concentration and exposure time of cells to the test item under study. However, the bacteria most commonly used in these reverse mutation assays do not possess the enzyme system which, in mammals, is known to convert promutagens into active DNA damaging metabolites necessary (Bradley *et al.*, 1981). In order to overcome this major drawback, an exogenous metabolic system was added in the form of mammalian microsome enzyme activation mixture.

The S9 liver microsomal fraction was prepared at BSL BIOSERVICE GmbH. Male Wistar rats were induced with Phenobarbital (80 mg/kg bodyweight) and β-naphtoflavone (100 mg/kg bodyweight) for three consecutive days by the oral route. The rats were humanely euthanized and the livers harvested. Livers were homogenized and then centrifuged at 9000 g for 20 minutes. The resulting supernatant containing the microsomes was frozen in ampoules of 2.0 and 4.5 mL and stored at ≤-75°C. The protein concentration in the S9 preparation (Lot: 140607 (Experiments I through IV) and Lot 291107 (Experiments III and IV)) were 34 mg/mL and 35 mg/mL, respectively.

The S9 cofactor solution preparation was performed according to Ames *et al.* (1973). Ice-cold sodium-ortho-phosphate buffer (pH 7.4, 100 mM) was added to sterilized pre-weighed reagents to give final concentrations of 8 mM MgCb, 33 mM KCI, 5 mM Glucose-6-phosphate, and 4 mM NADP in the S9 mix. This solution was mixed with the liver supernatant fluid (9.5 parts and 0.5 parts, respectively). During the experiment, the S9 mix was stored on ice. Quality control determinations were performed to verify the biological activity in the *S. typhimurium* assay and the sterility of the mix.

3.3 Reverse Mutation Assay

The test item was dissolved in ethanol and diluted prior to treatment. The solvent was compatible with the survival of the bacteria and the S9 activity. Positive and negative controls were included in each experiment. Strain specific positive controls were included in the assay, which demonstrated the effective performance of the test. Negative solvent controls, consisting of solvent or vehicle alone as well as untreated controls were treated in the same way as the treatment groups. Positive controls were tester strain specific (Table 1). The stability of the

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positive control substances in solution is unknown but a mutagenic response in the expected range is sufficient evidence of biological stability.

Table 1. Positive Controls Substances, Specific to *S. typhimurium* Strain, with and without Metabolic Activation

S. typhimurium Strain	Control	Supplier	Purity	Solvent	Concentration
Without metabolic activation					
TA 100, TA 1535	Sodium azide, NaN_3	Merck	≥99%	Aqua dest	10 µg/plate
TA 98, TA 1537	4-nitro-o-phenylene-diamine,4-NOPD	Fluka	>97%	DMSO	10 µg/plate
TA 102	Methyl methane sulfonate, MMS	Sigma	99.0%	Aqua dest	1 µg/plate
With metabolic activation					
TA 98, TA 100, TA 1535, TA 1537	2-aminoanthracene, 2-AA	Aldrich	96%	DMSO	2.5 µg/plate
TA 102	2-aminoanthracene, 2-AA	Aldrich	96%	DMSO	10 µg/plate

Notes: Aqua dest = top quality distilled water; DMSO = dimethylsulfoxide

3.3.1 Bacteria. Five strains of *S. typhimurium* were used (Table 2). The *Salmonella* tester strains TA 98, TA 100, TA 102 and TA 1535 were obtained from Xenometrix, San Diego, CA, USA. Tester strain TA 1537 was obtained from MOLTOX, Inc., NC, USA. Bacteria were stored as stock cultures in ampoules with nutrient broth (OXOID, Basingstoke, Hampshire, UK) supplemented with DMSO (dimethyl sulfoxide, approximately 8 percent volume/volume) over liquid nitrogen.

Table 2. *S. typhimurium* Strains and Characteristics

Strain	Histidine Mutation	Mutation Type
TA98	his D 3052	R-factor: frame shift mutations
TA 100	his G 46	R-factor: base-pair substitutions
TA 1535	his G 46	base-pair substitutions
TA 1537	his C 3076	frame shift mutations
TA 102	his G 428 (PAQ1)	R-factor: base-pair substitutions

All S. *typhimurium* strains contain mutations in the histidine operon, thereby imposing a requirement for histidine in the growth medium. They contain the deep rough (*rfa*) mutation, which deletes the polysaccharide side chain of the lipopolysaccharides of the bacterial cell surface. This increases cell permeability of larger substances. The other mutation is a deletion of the *uvr*B gene coding for the DNA excision repair system resulting in an increased sensitivity

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in detecting many mutagens. This deletion also includes the nitrate reductase (*chI*) and biotin (*bio*) genes (bacteria require biotin for growth).

The tester strains TA 98, TA 100 and TA 102 contain the R-factor plasmid, pkM 101. These strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains. pkM 101 increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in these organisms (Maron and Ames, 1983; Mortelmans and Zeiger, 2000).

The properties of the S. *typhimurium* strains with regard to membrane permeability, ampicillin- and tetracycline-resistance as well as normal spontaneous mutation rates are checked regularly as required by Ames *et al.* (1973). In this way it is ensured that the experimental conditions set up by Ames are fulfilled.

3.3.2 Preparation of Bacteria and Media. Samples of each tester strain were grown by culturing the bacteria for 12 hours at 38.5°C in nutrient broth to the late exponential or early stationary phase of growth (approximately 10^9 cells/mL). The nutrient medium consists of 8 g Nutrient Broth and 5 g NaCl per liter. A solution of 125 µL ampicillin (10 mg/mL) (TA 98, TA 100, TA 102) was added in order to retain the phenotypic characteristics of the strain.

Vogel-Bonner Medium E agar plates with 2 percent glucose used in the Ames test were prepared by BSL BIOSERVICE or provided by an appropriate supplier. Quality controls were performed. Vogel-Bonner Medium E agar plates contained 15 g agar, 20 mL Vogel-Bonner salts, and 50 mL glucose-solvent (40 percent) per liter. Vogel-Bonner-salts consist of 10 g $MgS0_4$ X 7 H_20; 100 g citric acid; 175 g $NaNH_4HP0_4$ x 4 H_20; and 500 g K_2HP0_4 per liter. The overlay agar contained 7.0 g agar; 6.0 g NaCl; 10.5 mg L-histidine x HCl x H_20; and 12.2 mg biotin per liter. Agars were sterilized at 121°C in an autoclave.

3.3.3 Exposure Concentration Determination. The toxicity of the test item was determined with tester strains TA 98 and TA 100 in a pre-experiment. Eight concentrations were tested for toxicity and induction of mutations with three plates each. The experimental conditions in this pre-experiment were the same as described below for the main Experiment I (plate incorporation test). F-T fuel was tested in the pre-experiment at 0.00316, 0.0100, 0.0316, 0.100, 0.316, 1.0, 2.5 and 5.0 µL/plate.

Test item concentrations to be applied in the main experiments were chosen according to the results of the pre-experiment, with a maximum concentration of 5.0 µL/plate. The concentration range covered two logarithmic decades. Two independent experiments were performed with the following concentrations. Experiment I tested 0.0316, 0.100, 0.316, 1.0, 2.5 and 5.0 µL/plate. Experiment II used 0.1875, 0.375, 0.75, 1.5, 3.0 and 5.0 µL/plate. As the results of the pre-experiment were in accordance with the criteria described above, these were reported as a part of Experiment I.

3.3.4 Plate Incorporation Method. For the plate incorporation method, 100 µL test solution (each dose level, solvent control, negative control or reference mutagen solution (positive control)); 500 µL S9 mix (for testing with metabolic activation) or S9 mix substitution buffer

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(for testing without metabolic activation); 100 µL bacteria suspension preparation preculture of the strain); and 2000 µL overlay agar were mixed in a test tube and poured over the surface of a minimal agar plate. For each strain and dose level, including the controls, three plates were used. After solidification, the plates were inverted and incubated at 37°C for at least 48 hours in the dark.

3.3.5 Cytotoxicity. The colonies were counted using a ProtoCOL counter (Meintrup DWS Laborgerate GmbH, Lähden, Germany). If precipitation of the test item precluded automatic counting, the revertant colonies were counted by hand. In addition, tester strains with a low spontaneous mutation frequency such as TA 1535 and TA 1537 were counted manually. Cytotoxicity was determined either by a clearing or a diminution of the background lawn (indicated as "B" in the result tables) or a reduction in the number of revertants down to a mutation factor of approximately ≤0.5 in relation to the solvent control.

A test was considered acceptable, if, for each strain:
- the bacteria demonstrate their typical responses to ampicillin (TA 98, TA 100, TA 102)
- the control plates with and without S9 mix are within the ranges shown in Table 3 (mean values of the spontaneous reversion frequency are within the historical control data range)
- corresponding background growth on negative control, solvent control and test plates is observed, or
- the positive controls show a distinct enhancement of revertant rates over the control plate.

Table 3. Control Reverse Mutation Ranges by Strain, with and without Activation

Strain	-S9	+S9
TA 98	18 - 54	16 - 71
TA 100	75 -171	83 – 168
TA 1535	6 - 30	6 – 31
TA 1537	5 - 31	6 – 36
TA 102	166 - 394	153 - 594

3.3.6 Evaluation of Mutagenicity. The Mutation Factor is calculated by dividing the mean value of the revertant counts by the mean values of the solvent control (the exact, not the rounded values, are used for calculation). A test item is considered as mutagenic if a clear and dose-related increase in the number of revertants occurs and/or a biologically relevant positive response for at least one of the dose groups occurs in at least one tester strain with or without metabolic activation.

A biologically relevant increase is strain dependent. In tester strains TA 100 and TA 102, the number of reversions must be at least twice as high as the solvent control. In tester strains TA 98, TA 1535 and TA 1537, the number of reversions should be at least three times higher than the reversion rate of the solvent control (Kier *et al.*, 1986).

According to Organisation for Economic Co-operation and Development (OECD) guidelines (1997a), the biological relevance of the results is the criterion for the interpretation of results. A

statistical evaluation of the results is not regarded as necessary. A test item producing neither a dose related increase in the number of revertants nor a reproducible biologically relevant positive response at any of the dose groups is considered to be non-mutagenic in this system.

3.4 Chromosome Aberration Assay

The test item F-T jet fuel was dissolved in 500 µL/mL ethanol; no precipitation of the test item was indicated. During this assay, 1 percent of this solution was diluted in cell culture medium (RPMI 1640) prior to treatment. The solvent was compatible with the survival of the cells and the S9 activity. After dilution with cell culture medium, precipitation of the test item appeared in a concentration of 5 µL/mL.

Positive and negative controls were included. Negative controls, consisting of vehicle alone and treated in the same way as the treatment groups were included. Concurrent negative and/or solvent controls were performed. The positive control, without metabolic activation, was ethylmethanesulfonate (EMS, Sigma, purity > 98 percent) dissolved in nutrient medium at final concentrations of 400 and 600 µg/mL. These solutions were prepared on the day of the experiment. The positive control with metabolic activation was cyclophosphamide (CPA, Sigma, purity ≥ 98 percent) dissolved in nutrient medium at a final concentration of 7.5 µg/mL. The stability of CPA at room temperature was good. The stability of the positive control substance in solution was proven by the mutagenic response in the expected range. At 25°C only 3.5 percent of its potency was lost after 24 hours (Gallelli, 1967). The solution was stored in aliquots at 15°C.

Blood samples were obtained from healthy donors not receiving medication. In each experiment, blood was collected only from a single donor to reduce inter-individual variability. Blood samples were drawn by venous puncture and collected in heparinized tubes. Before use, the blood was stored under sterile conditions at 4°C for a maximum of 4 hours.

3.4.1 Pre-Experiment for Toxicity and Exposure Concentrations. According to the relevant guidelines, the highest recommended dose is 5 mg/mL, 5 µL/mL or 10 mM, whichever is the lowest. The highest dose group evaluated in the pre-experiment was 5 µL/mL. The relative mitotic index was used as a parameter for toxicity. The concentrations evaluated in the main experiment were based on the results obtained in the pre-experiment.

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Table 4. Pre-Experiment for Cytotoxicity

Dose Group	Concentration [µL/mL]	Mitotic Index	relative [%]
without metabolic activation			
C	0	71	99
S	0	72	100
1	0.008	62	86
2	0.016	58	81
3	0.031	64	89
4	0.063	44	61
5	0.125	40	56
6	0.25	40	56
7	0.5	42	58
8	1.0	32	44
9	2.5	38	53
10	5	32	44
with metabolic activation			
C	0	57	124
S	0	46	100
1	0.008	64	139
2	0.016	78	170
3	0.031	58	126
4	0.063	62	135
5	0.125	54	117
6	0.25	53	115
7	0.5	75	163
8	1.0	44	96
9	2.5	52	113
10	5	53	115

The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the control.
C: Negative Control
S: Solvent Control (Ethanol)

Duplicate cultures were treated at each concentration. The selection of the concentrations used in Experiments III and IV were based on data from the pre-experiment. In Experiment III, concentrations used with metabolic activation were 0.125, 0.25, 0.5, 1.0, 2.5 and 5 µL/mL, and without metabolic activation were 0.0016, 0.005, 0.016, 0.05, 0.16, 0.50, 1.58 and 5 µL/mL. In Experiment IV, concentrations used with metabolic activation were 0.5, 1, 2, 3, 4 and 5 µL/mL, and without metabolic activation were 0.00016, 0.0005, 0.0016, 0.005, 0.016, 0.05, 0.16, 0.50, 1.58 and 5 µL/mL.

The cells were treated in Experiment III (with and without metabolic activation) for 4 hours with the test item. The metaphases were prepared 24 hours after the treatment. In Experiment IV with metabolic activation, the cells were treated for 4 hours and prepared 24 hours after the treatment. In Experiment IV without metabolic activation, the cells were treated for 24 hours

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and prepared at the end of the treatment. The dose group selection for microscopic analyses of chromosomal aberrations was based on the mitotic index in accordance with the guidelines.

The following concentrations were selected in the main experiments for the microscopic analysis: Experiment III with metabolic activation (4 hours treatment, 24 hours preparation interval, 1.0, 2.5 and 5 µL/mL) and without metabolic activation (4 hours treatment, 24 hours preparation interval, 0.16, 0.50, 1.58 and 5 µL/mL); Experiment IV with metabolic activation (4 hours treatment, 24 hours preparation interval, 3, 4 and 5 µL/mL) and without metabolic activation (24 hours treatment, 24 hours preparation interval, 0.50, 1.58 and 5 µL/mL). At least three analyzable concentrations of the test item were used for the 24 hours preparation.

3.4.2 Culture Initiation, Treatment and Preparation. Blood cell cultures were set up within 4 hours after collection. The following volumes were added to the plastic culture vessels: 8.45 mL culture medium, 0.90 mL whole blood, 0.50 mL PHA, 0.10 mL antibiotic solution, and 0.05 mL heparin. All incubations were done at 37°C in humidified atmosphere with 5 percent CO_2.

- *Experiment III. Short time exposure 4 hours (with and without S9 mix):* After 48 hours, the culture medium was replaced with serum-free medium containing the test item (without metabolic activation) or serum-free medium containing the test item with 50 µL/mL S9 mix (with metabolic activation). After 4 hours, the cells were spun down by centrifugation at 1000 rotations per minute for 5 minutes. The supernatant with the dissolved test item was discarded and the cells were resuspended in PBS. The washing procedure was repeated once as described. After washing, the cells were resuspended in complete cell culture medium. The cells were prepared 24 hours after the beginning of the treatment.

- *Experiment IV. Long time exposure 24 hours (without S9 mix):* After 48 hours the culture medium was replaced with complete medium (with 15 percent FCS) containing the test item without S9 mix. This medium was not changed until preparation of the cells 24 hours after the beginning of the treatment.

- *Experiment IV. Exposure time with metabolic activation:* The cells were treated as described for Experiment III (with metabolic activation). The cells were prepared 24 hours after the beginning of the treatment.

Two to three hours before harvesting, Colcemid was added to the cultures (final concentration 0.2 µg/mL). The cultures were harvested by centrifugation 24 hours after beginning of treatment. The supernatant was discarded and the cells were resuspended in approximately 5 mL hypotonic solution (0.4 percent KCl). The cell suspension was incubated at room temperature for 20 minutes. After removal of the hypotonic solution by centrifugation, the cells were fixed with 3 + 1 methanol + glacial acetic acid. The fixation procedure was repeated twice. Slides were prepared by dropping the cell suspension on to a clean microscopic slide. The cells were then stained with Giemsa or in accordance with the fluorescent plus Giemsa technique.

3.4.3 Proliferation Index. The negative control and the highest dose group evaluated were treated in the presence of BrdU to measure the proliferation index and/or replication time of the

cultured lymphocytes. The proliferation index was determined by scoring the number of first, second and third metaphases in 100 cells per culture. The proliferation index (PI) was calculated as:

$$PI = \frac{1(\% \text{ cells in M1}) + 2(\% \text{ cells in M2}) + 3(\% \text{ cells in M3})}{100}$$

(1)

Where M1 = first mitosis, M2 = second mitosis, M3 = third mitosis, with respect to the beginning of the exposure.

3.4.4 Analysis of Metaphase Cells. All slides, including those of positive and negative controls, were independently coded before microscopic analysis. Evaluation of the cultures was performed (according to the standard protocol of the "Arbeitsgruppe der Industrie, Cytogenetik" (Engelhardt, 1987)) using OLYMPUS microscopes with 100x oil immersion objectives. Structural chromosomal aberrations including breaks, fragments, deletions, exchanges and chromosomal disintegrations were recorded. Gaps were recorded as well, but not included in the calculation of the aberration rates. The definition of a gap was as follows: an achromatic region (occurring in one or both chromatids) independent of its width. The remaining visible chromosome regions should not be dislocated longitudinally or laterally. At least 200 well spread metaphases per concentration and control were scored for cytogenetic damage. Metaphases with 46±2 centromer regions were included in the analysis (Engelhardt, 1987; Scott *et al.*, 1990). To describe a cytotoxic effect, the mitotic index (percent cells in mitosis) was determined. Additionally the number of polyploidy cells was scored.

3.4.5 Data Recording. The data generated were recorded in the raw data file. The results are presented in tables, including experimental groups with the test item plus negative and positive controls. The experimental unit is the cell and therefore, the percentage of cells with structural aberration was evaluated. Different types of chromosome aberrations are listed with their numbers of frequencies for experimental and control groups. Gaps were recorded separately and reported but generally not included in the aberration frequency. Concurrent measurements of cytotoxicity were also recorded.

3.4.6 Evaluation of Results. The chromosomal aberration assay is considered acceptable if it meets the following criteria. The number of aberrations found in the negative and/or solvent controls must fall within the range of historical laboratory control data: 0.0 - 4.0 percent. Additionally, the positive control substance should produce biologically relevant increases in the number of cells with structural chromosome aberrations.

There are several criteria for determining a positive result. A clear and dose-related increase in the number of cells with aberrations must be present. A biologically relevant response is observed for at least one of the dose groups, which is higher than the laboratory negative control range (up to 4.0 percent aberrant cells).

According to the OECD guidelines (1997b), the biological relevance of the results will be the criterion for the interpretation of results, a statistical evaluation of the results is not regarded as necessary. However, for the interpretation of the data, both biological and, if evaluated,

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statistical significance should be considered together. A test item is considered to be negative if there is no biologically relevant increase in the percentages of aberrant cells above concurrent control levels, at any dose group.

4.0 RESULTS AND DISCUSSION

4.1 Reverse Mutation Assay

Pre-experimental testing for toxicity was carried out in two strains of *S. typhimurium* (TA 98 and TA 100), with and without S9 activation. Toxicity may be detected by a clearing or rather diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤0.5 in relation to the solvent control. None of the pre-experiment conditions tested resulted in toxicity, according to this definition (Table 5).

The test item F-T jet fuel was investigated for its potential to induce gene mutations according to the plate incorporation test (Experiment I and II, Tables 6 and 7) using *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 102. In two independent experiments, several concentrations of the test item were used. Each assay was conducted with and without metabolic activation. The concentrations, including the controls, were tested in triplicate. No precipitation of the test item on the agar plates was observed in any of the five tester strains used in Experiment I and II (with and without metabolic activation). However, a clouding of the S9 mix and the S9 substitution buffer after addition of the test item solution was noted at a concentration of 0.316 µL/plate and higher (with and without metabolic activation) in Experiment I and at a concentration of 0.375 µL/plate and higher (with and without metabolic activation) in Experiment II.

No toxic effects of the test item were noted in any of the five tester strains used up to the highest dose group evaluated (with and without metabolic activation) in Experiment I and II. No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with F-T jet fuel at any concentration level, neither in the presence nor absence of metabolic activation in Experiment I and II, as compared to solvent controls, positive controls or historical data (Appendix A). The reference mutagens induced a distinct increase of revertant colonies, indicating the validity of the experiments.

Table 5. Results of Pre-Experiment Toxicity Testing, with and without Activation

Substance	Dose (µL/plate)	TA 98 Mutation Factor [toxicity]*		TA 100 Mutation Factor [toxicity]*	
		without S9	with S9	without S9	with S9
Solvent Control (EtOH)		1.0	1.0	1.0	1.0
4-NOPD	10.0 µg	17.6	-	-	-
NaN₃	10.0 µg	-	-	6.1	-
2-AA	2.5 µg	-	37.1	-	17.6
Test Item	0.00316	1.0	0.9	0.8	1.0
	0.0100	0.9	0.9	1.1	1.1
	0.0316	0.9	0.8	0.9	1.0
	0.100	0.8	0.9	1.0	0.9
	0.316	0.9	0.9	0.8	0.9
	1.0	0.9	0.9	0.8	0.9
	2.5	0.9	0.8	0.8	0.8
	5.0	0.7	1.0	0.8	0.8

* [toxicity parameter]: B= Background lawn reduced; N= No background lawn

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Table 6. Results of Experiment I Plate-Incorporation Test by Strain, with and without Activation

Tester Strain: TA 98   Experiment: 1

| Treatment | Dose/plate | REVERTANT COLONIES PER PLATE | | | | | | MUTATION FACTOR | |
| | | Without activation (-S9) | | | With activation (+S9) | | | | |
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		18 24 24	22	3.5	26 28 36	30	5.3	0.8	0.8
EtOH		30 22 28	27	4.2	38 40 39	39	1.0	1.0	1.0
Test Item	0.0316 µL	26 25 19	23	3.8	30 34 34	33	2.3	0.9	0.8
Test Item	0.100 µL	21 19 26	22	3.6	27 37 44	36	8.5	0.8	0.9
Test Item	0.316 µL	26 31 15	24	8.2	34 35 34	34	0.6	0.9	0.9
Test Item	1.0 µL	29 18 24	24	5.5	45 26 35	35	9.5	0.9	0.9
Test Item	2.5 µL	23 19 27	23	4.0	32 41 20	31	10.5	0.9	0.8
Test Item	5.0 µL	22 22 14	19	4.6	41 36 43	40	3.6	0.7	1.0
4-NOPD	10 µg	415 513 483	470	50.2	/ / /	/	/	17.6	/
2-AA	2.5 µg	/ / /	/	/	1344 1571 1422	1446	115.3	/	37.1

SD: Standard deviation   P: Precipitation
B: Background lawn reduced   C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

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Table 6. Results of Experiment I Plate-Incorporation Test (continued)

Tester Strain: TA 100   Experiment: 1

| Treatment | Dose/plate | REVERTANT COLONIES PER PLATE | | | | | | MUTATION FACTOR | |
| | | Without activation (-S9) | | | With activation (+S9) | | | | |
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		103 93 96	97	5.1	128 133 126	129	3.6	1.1	1.2
EtOH		86 105 86	92	11.0	96 116 106	106	10.0	1.0	1.0
Test Item	0.0316 µL	72 107 70	83	20.8	94 122 111	109	14.1	0.9	1.0
Test Item	0.100 µL	85 88 94	89	4.6	101 106 91	99	7.6	1.0	0.9
Test Item	0.316 µL	67 62 96	75	18.4	90 102 91	94	6.7	0.8	0.9
Test Item	1.0 µL	68 79 78	75	6.1	90 103 85	93	9.3	0.8	0.9
Test Item	2.5 µL	63 91 80	78	14.1	99 81 78	86	11.4	0.8	0.8
Test Item	5.0 µL	71 85 69	75	8.7	93 83 84	87	5.5	0.8	0.8
NaN$_3$	10 µg	582 577 534	564	26.4	/ / /	/	/	6.1	/
2-AA	2.5 µg	/ / /	/	/	1771 1818 2000	1863	121.0	/	17.6

SD: Standard deviation   P: Precipitation
B: Background lawn reduced   C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Table 6. Results of Experiment I Plate-Incorporation Test (continued)

Tester Strain: TA 1535   Experiment: 1

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		9 7 11	9	2.0	7 5 9	7	2.0	1.8	0.6
EtOH		3 7 5	5	2.0	10 14 11	12	2.1	1.0	1.0
Test Item	0.0316 µL	3 3 8	5	2.9	11 10 10	10	0.6	0.9	0.9
Test Item	0.100 µL	5 11 11	9	3.5	7 9 11	9	2.0	1.8	0.8
Test Item	0.316 µL	8 6 13	9	3.6	12 16 6	11	5.0	1.8	1.0
Test Item	1.0 µL	9 9 15	11	3.5	19 12 10	14	4.7	2.2	1.2
Test Item	2.5 µL	11 11 9	10	1.2	17 13 12	14	2.6	2.1	1.2
Test Item	5.0 µL	4 4 5	4	0.6	11 11 10	11	0.6	0.9	0.9
NaN₃	10 µg	717 810 911	813	97.0	/ / /	/	/	162.5	/
2-AA	2.5 µg	/ / /	/	/	84 91 85	87	3.8	/	7.4

SD: Standard deviation   P: Precipitation
B: Background lawn reduced   C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Table 6. Results of Experiment I Plate-Incorporation Test (continued)

Tester Strain: TA 1537   Experiment: 1

| Treatment | Dose/plate | REVERTANT COLONIES PER PLATE | | | | | | MUTATION FACTOR | |
| | | Without activation (-S9) | | | With activation (+S9) | | | | |
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		12 11 11	11	0.6	21 17 9	16	6.1	1.3	1.6
EtOH		7 10 9	9	1.5	10 14 6	10	4.0	1.0	1.0
Test Item	0.0316 µL	6 4 9	6	2.5	10 11 14	12	2.1	0.7	1.2
Test Item	0.100 µL	4 10 12	9	4.2	8 12 10	10	2.0	1.0	1.0
Test Item	0.316 µL	9 9 8	9	0.6	10 7 11	9	2.1	1.0	0.9
Test Item	1.0 µL	8 9 7	8	1.0	19 20 7	15	7.2	0.9	1.5
Test Item	2.5 µL	7 10 7	8	1.7	13 15 8	12	3.6	0.9	1.2
Test Item	5.0 µL	8 1 13	7	6.0	15 12 9	12	3.0	0.8	1.2
4-NOPD	40 µg	159 195 149	168	24.2	/ / /	/	/	19.3	/
2-AA	2.5 µg	/ / /	/	/	391 382 370	381	10.5	/	38.1

SD: Standard deviation
B: Background lawn reduced
N: No background lawn

P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

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Table 6. Results of Experiment I Plate-Incorporation Test (continued)

Tester Strain: TA 102   Experiment: 1

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)			-S9	+S9
		Counts	Mean	SD	Counts	Mean	SD		
A. dest.		258 271 290	273	16.1	273 272 331	292	33.8	1.1	1.0
EtOH		253 240 259	251	9.7	289 295 308	297	9.7	1.0	1.0
Test Item	0.0316 µL	192 287 275	251	51.7	187 272 270	243	48.5	1.0	0.8
Test Item	0.100 µL	286 310 264	287	23.0	243 239 245	242	3.1	1.1	0.8
Test Item	0.316 µL	274 256 238	256	18.0	292 364 352	336	38.6	1.0	1.1
Test Item	1.0 µL	221 244 302	256	41.7	314 267 309	297	25.8	1.0	1.0
Test Item	2.5 µL	203 203 249	218	26.6	320 312 282	305	20.0	0.9	1.0
Test Item	5.0 µL	223 210 234	222	12.0	305 352 318	325	24.3	0.9	1.1
MMS	1 µL	1491 1263 1368	1374	114.1	/ / /	/	/	5.5	/
2-AA	10 µg	/ / /	/	/	720 890 765	792	88.1	/	2.7

SD: Standard deviation
B: Background lawn reduced
N: No background lawn

P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

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Table 7. Results of Experiment II Plate-Incorporation Test by Strain, with and without Activation

Tester Strain: TA 98   Experiment: 2

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		17 27 18	21	5.5	30 19 30	26	6.4	0.8	0.6
EtOH		25 22 29	25	3.5	32 43 48	41	8.2	1.0	1.0
Test Item	0.1875 µL	12 27 22	20	7.6	38 36 26	33	6.4	0.8	0.8
Test Item	0.375 µL	20 19 23	21	2.1	49 39 31	40	9.0	0.8	1.0
Test Item	0.75 µL	30 21 16	22	7.1	29 23 35	29	6.0	0.9	0.7
Test Item	1.5 µL	25 26 20	24	3.2	28 31 37	32	4.6	0.9	0.8
Test Item	3.0 µL	23 31 16	23	7.5	29 29 25	28	2.3	0.9	0.7
Test Item	5.0 µL	22 20 22	21	1.2	32 31 35	33	2.1	0.8	0.8
4-NOPD	10 µg	658 702 900	753	128.9	/ / /	/	/	29.7	/
2-AA	2.5 µg	/ / /	/	/	3119 3118 3011	3083	62.1	/	75.2

SD: Standard deviation   P: Precipitation
B: Background lawn reduced   C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Table 7. Results of Experiment II Plate-Incorporation Test (continued)

Tester Strain: TA 100   Experiment: 2

| Treatment | Dose/plate | REVERTANT COLONIES PER PLATE | | | | | | MUTATION FACTOR | |
| | | Without activation (-S9) | | | With activation (+S9) | | | | |
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		110 130 107	116	12.5	100 89 109	99	10.0	1.1	1.0
EtOH		107 112 93	104	9.8	107 104 90	100	9.1	1.0	1.0
Test Item	0.1875 µL	79 66 107	84	21.0	94 104 86	95	9.0	0.8	0.9
Test Item	0.375 µL	93 84 116	98	16.5	101 71 87	86	15.0	0.9	0.9
Test Item	0.75 µL	86 100 86	91	8.1	83 86 69	79	9.1	0.9	0.8
Test Item	1.5 µL	72 78 90	80	9.2	96 84 68	83	14.0	0.8	0.8
Test Item	3.0 µL	79 72 81	77	4.7	81 76 80	79	2.6	0.7	0.8
Test Item	5.0 µL	75 97 69	80	14.7	73 81 79	78	4.2	0.8	0.8
NaN$_3$	10 µg	519 572 652	581	67.0	/ / /	/	/	5.6	/
2-AA	2.5 µg	/ / /	/	/	2154 2123 2422	2233	164.4	/	22.3

SD: Standard deviation   P: Precipitation
B: Background lawn reduced   C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

Table 7. Results of Experiment II Plate-Incorporation Test (continued)

Tester Strain: TA 1535   Experiment: 2

| Treatment | Dose/plate | REVERTANT COLONIES PER PLATE | | | | | | MUTATION FACTOR | |
| | | Without activation (-S9) | | | With activation (+S9) | | | | |
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		11 13 6	10	3.6	12 12 9	11	1.7	1.2	0.7
EtOH		11 8 7	9	2.1	15 18 12	15	3.0	1.0	1.0
Test Item	0.1875 µL	5 11 7	8	3.1	8 14 11	11	3.0	0.9	0.7
Test Item	0.375 µL	11 6 6	8	2.9	15 6 7	9	4.9	0.9	0.6
Test Item	0.75 µL	8 8 7	8	0.6	18 19 11	16	4.4	0.9	1.1
Test Item	1.5 µL	8 9 5	7	2.1	15 18 12	15	3.0	0.8	1.0
Test Item	3.0 µL	12 7 9	9	2.5	17 13 15	15	2.0	1.1	1.0
Test Item	5.0 µL	5 9 10	8	2.6	13 13 11	12	1.2	0.9	0.8
NaN$_3$	10 µg	808 670 800	759	77.5	/ / /	/	/	87.6	/
2-AA	2.5 µg	/ / /	/	/	111 108 99	106	6.2	/	7.1

SD: Standard deviation   P: Precipitation
B: Background lawn reduced   C: Contamination
N: No background lawn

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

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Table 7. Results of Experiment II Plate-Incorporation Test (continued)

Tester Strain: TA 1537

Experiment: 2

| Treatment | Dose/plate | REVERTANT COLONIES PER PLATE | | | | | | MUTATION FACTOR | |
| | | Without activation (-S9) | | | With activation (+S9) | | | | |
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		10 11 12	11	1.0	13 12 11	12	1.0	1.1	1.1
EtOH		8 8 14	10	3.5	10 15 7	11	4.0	1.0	1.0
Test Item	0.1875 µL	9 9 13	10	2.3	4 13 10	9	4.6	1.0	0.8
Test Item	0.375 µL	4 9 12	8	4.0	16 6 14	12	5.3	0.8	1.1
Test Item	0.75 µL	6 12 12	10	3.5	11 7 7	8	2.3	1.0	0.8
Test Item	1.5 µL	5 10 11	9	3.2	9 8 14	10	3.2	0.9	1.0
Test Item	3.0 µL	15 11 12	13	2.1	14 7 9	10	3.6	1.3	0.9
Test Item	5.0 µL	13 5 6	8	4.4	9 10 6	8	2.1	0.8	0.8
4-NOPD	40 µg	160 120 /	140	28.3	/ / /	/	/	14.0	/
2-AA	2.5 µg	/ / /	/	/	323 409 403	378	48.0	/	35.5

SD: Standard deviation
B: Background lawn reduced
N: No background lawn

P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

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Table 7. Results of Experiment II Plate-Incorporation Test (continued)

Tester Strain: TA 102

Experiment: 2

Treatment	Dose/plate	REVERTANT COLONIES PER PLATE						MUTATION FACTOR	
		Without activation (-S9)			With activation (+S9)				
		Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
A. dest.		271 311 334	305	31.9	333 350 346	343	8.9	1.0	0.9
EtOH		289 284 301	291	8.7	389 367 370	375	11.9	1.0	1.0
Test Item	0.1875 µL	305 306 341	317	20.5	379 399 399	392	11.5	1.1	1.0
Test Item	0.375 µL	276 336 287	300	31.9	419 381 397	399	19.1	1.0	1.1
Test Item	0.75 µL	349 354 356	353	3.6	394 383 396	391	7.0	1.2	1.0
Test Item	1.5 µL	310 314 325	316	7.8	354 284 179	272	88.1	1.1	0.7
Test Item	3.0 µL	341 317 304	321	18.8	363 279 229	290	67.7	1.1	0.8
Test Item	5.0 µL	182 224 261	222	39.5	381 409 413	401	17.4	0.8	1.1
MMS	1 µL	1960 1904 1748	1871	109.9	/ / /	/	/	6.4	/
2-AA	10 µg	/ / /	/	/	1324 1307 1460	1364	83.9	/	3.6

SD: Standard deviation
B: Background lawn reduced
N: No background lawn

P: Precipitation
C: Contamination

$$\text{Mutation factor} = \frac{\text{mean revertants (test item)}}{\text{mean revertants (solvent control)}}$$

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4.2 Chromosomal Aberration Assay

The test item F-T jet fuel was investigated for a possible potential to induce structural chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 homogenate. The chromosomes were prepared 24 hours after start of treatment with the test item. The treatment interval in Experiment III was 4 hours with and without metabolic activation (Tables 8 and 9, respectively). The treatment interval in Experiment IV was 4 hours with metabolic activation and 24 hours without metabolic activation (Tables 10 and 11, respectively). Two parallel cultures were set up per dose group. Per culture, 100 metaphases were scored for structural chromosomal aberrations. A summary of the results is presented in Tables 12 and 13. Precipitation of the test item was noted with and without metabolic activation after the incubation at a concentration of 5 µL/mL.

Table 8. Experiment III. Structural Chromosomal Aberrations, without Metabolic Activation: 4 hours Treatment, 24 hours Fixation Period

Dose Group	Concentration [µL/mL]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Chromatid types b	Chromatid types f	Chromatid types d	Chromatid types ex	Chromosome types ib	Chromosome types if	Chromosome types id	Chromosome types cx	Other ma	Other cd
C	0	1	100	0	3	2	1	0	2	0	0	0	0	0	0	0	0	0
		2	100	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0
		total	200	0	6	3	3	0	3	0	0	0	0	0	0	0	0	0
S	0	1	100	0	3	2	1	0	1	1	0	0	0	0	0	0	0	0
		2	100	0	2	1	1	0	1	0	0	0	0	0	0	0	0	0
		total	200	0	5	3	2	0	2	1	0	0	0	0	0	0	0	0
5	0.16	1	100	1	2	1	1	0	1	0	0	0	0	0	0	0	0	0
		2	100	0	5	3	2	0	1	2	0	0	0	0	0	0	0	0
		total	200	1	7	4	3	0	2	2	0	0	0	0	0	0	0	0
6	0.50	1	100	0	2	1	1	0	0	1	0	0	0	0	0	0	0	0
		2	100	0	5	1	4	0	1	0	0	0	0	0	0	0	0	0
		total	200	0	7	2	5	0	1	1	0	0	0	0	0	0	0	0
7	1.58	1	100	0	5	1	3	1	1	0	0	0	0	0	0	0	0	0
		2	100	1	2	1	0	1	0	1	0	0	0	0	0	0	0	0
		total	200	1	7	2	3	2	1	1	0	0	0	0	0	0	0	0
8	5	1	100	0	5	2	2	1	0	2	0	0	0	0	0	0	0	0
		2	100	0	4	1	3	0	1	0	0	0	0	0	0	0	0	0
		total	200	0	9	3	5	1	1	2	0	0	0	0	0	0	0	0
EMS	600 µg/mL	1	100	0	15	12	8	0	6	2	0	8	0	1	0	0	0	0
		2	100	0	15	9	6	1	7	0	0	3	0	0	0	0	0	0
		total	200	0	30	21	14	1	13	2	0	11	0	1	0	0	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
EMS: Positive Control

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

Distribution A: Approved for public release; distribution unlimited.

Table 9. Experiment III. Structural Chromosomal Aberrations, with Metabolic Activation: 4 hours Treatment, 24 hours Fixation Period

Dose Group	Concentration [µL/mL]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Chromatid types b	f	d	ex	Chromosome types ib	if	id	cx	Other ma	cd
C	0	1	100	0	4	1	4	0	1	0	0	0	0	0	0	0	0	0
		2	100	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0
		total	200	0	7	2	6	0	2	0	0	0	0	0	0	0	0	0
S	0	1	100	0	4	2	2	0	1	1	0	0	0	0	0	0	0	0
		2	100	0	5	2	4	1	1	1	0	0	0	0	0	0	0	0
		total	200	0	9	4	6	1	2	2	0	0	0	0	0	0	0	0
4	1.0	1	100	1	3	0	2	2	0	0	0	0	0	0	0	0	0	0
		2	100	1	9	3	5	1	3	0	0	0	0	0	0	0	0	0
		total	200	2	12	3	7	3	3	0	0	0	0	0	0	0	0	0
5	2.5	1	100	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
		2	100	0	2	1	1	0	0	0	0	1	0	0	0	0	0	0
		total	200	0	3	1	2	0	0	0	0	1	0	0	0	0	0	0
6	5	1	100	0	3	2	1	0	2	0	0	0	0	0	0	0	0	0
		2	100	0	4	1	2	1	0	0	0	0	0	1	0	0	0	0
		total	200	0	7	3	3	1	2	0	0	0	0	1	0	0	0	0
CPA	7.5 µg/mL	1	100	0	11	9	3	1	7	0	2	3	0	0	0	0	0	0
		2	100	0	15	11	4	0	7	2	0	7	1	1	0	0	0	0
		total	200	0	26	20	7	1	14	2	2	10	1	1	0	0	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
CPA: Positive Control

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

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Table 10. Experiment IV. Structural Chromosomal Aberrations, without Metabolic Activation: 24 hours Treatment, 24 hours Fixation Period

Dose Group	Concentration [µL/mL]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Chromatid types b	f	d	ex	Chromosome types ib	if	id	cx	Other ma	cd
C	0	1	100	1	7	2	4	1	1	1	0	0	0	0	0	0	0	0
		2	100	0	3	1	1	1	1	0	0	0	0	0	0	0	0	0
		total	200	1	10	3	5	2	2	1	0	0	0	0	0	0	0	0
S	0	1	100	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0
		2	100	0	7	1	6	0	1	0	0	0	0	0	0	0	0	0
		total	200	0	10	2	8	0	2	0	0	0	0	0	0	0	0	0
8	0.50	1	100	0	10	4	7	1	2	2	0	0	0	0	0	0	0	0
		2	100	1	2	1	1	0	0	1	0	0	0	0	0	0	0	0
		total	200	1	12	5	8	1	2	3	0	0	0	0	0	0	0	0
9	1.58	1	100	0	3	1	2	0	1	0	0	0	0	0	0	0	0	0
		2	100	0	6	3	2	1	1	2	0	0	0	0	0	0	0	0
		total	200	0	9	4	4	1	2	2	0	0	0	0	0	0	0	0
10	5	1	100	0	6	1	5	0	1	0	0	0	0	0	0	0	0	0
		2	100	0	5	3	2	0	3	0	0	0	0	0	0	0	0	0
		total	200	0	11	4	7	0	4	0	0	0	0	0	0	0	0	0
EMS	400 µg/mL	1	100	0	9	9	2	0	4	1	1	4	0	0	0	0	0	0
		2	100	0	14	12	5	0	10	1	0	2	0	0	0	0	0	0
		total	200	0	23	21	7	0	14	2	1	6	0	0	0	0	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
EMS: Positive Control

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

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Table 11. Experiment IV. Structural Chromosomal Aberrations, with Metabolic Activation: 4 hours Treatment, 24 hours Fixation Period

Dose Group	Concentration [µL/mL]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps g	Gaps ig	Chromatid types b	Chromatid types f	Chromatid types d	Chromatid types ex	Chromosome types ib	Chromosome types if	Chromosome types id	Chromosome types cx	Other ma	Other cd
C	0	1	100	0	9	3	10	0	3	0	0	0	0	0	0	0	0	0
		2	100	0	4	2	2	0	1	1	0	0	0	0	0	0	0	0
		total	200	0	13	5	12	0	4	1	0	0	0	0	0	0	0	0
S	0	1	100	0	3	2	1	0	0	1	0	0	1	0	0	0	0	0
		2	100	0	6	1	4	1	1	0	0	0	0	0	0	0	0	0
		total	200	0	9	3	5	1	1	1	0	0	1	0	0	0	0	0
4	3	1	100	0	5	2	3	1	1	0	0	1	0	0	0	0	0	0
		2	100	0	3	2	1	0	2	0	0	0	0	0	0	0	0	0
		total	200	0	8	4	4	1	3	0	0	1	0	0	0	0	0	0
5	4	1	100	0	3	0	3	0	0	0	0	0	0	0	0	0	0	0
		2	100	0	4	1	3	1	2	0	0	0	0	0	0	0	0	0
		total	200	0	7	1	6	1	2	0	0	0	0	0	0	0	0	0
6	5	1	100	0	6	2	4	1	2	0	0	0	0	0	0	0	0	0
		2	100	0	3	1	2	0	0	0	0	0	1	0	0	0	0	0
		total	200	0	9	3	6	1	2	0	0	0	1	0	0	0	0	0
CPA	7.5 µg/mL	1	100	0	10	10	1	0	4	3	0	3	0	0	0	0	0	0
		2	100	0	13	11	3	0	4	3	0	6	0	0	0	0	0	0
		total	200	0	23	21	4	0	8	6	0	9	0	0	0	0	0	0

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
CPA: Positive Control

(abbreviations: g – gap; ig – iso-gap; b – break; ib – iso-break; f – fragment; if – iso-fragment; d – deletion; id – iso-deletion; ma – multiple aberration; ex – chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

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Table 12. Summary of Aberration Rates for Experiment III

Dose Group	Concen-tration [μL/mL]	Treatment Time	Fixation Interval	mean % aberrant cells	
				incl. Gaps	excl. Gaps
without metabolic activation					
C	0	4 h	24 h	3.0	1.5
S	0	4 h	24 h	2.5	1.5
5	0.16	4 h	24 h	3.5	2.0
6	0.50	4 h	24 h	3.5	1.0
7	1.58	4 h	24 h	3.5	1.0
8	5	4 h	24 h	4.5	1.5
EMS	600 μg/mL	4 h	24 h	15.0	10.5
with metabolic activation					
C	0	4 h	24 h	3.5	1.0
S	0	4 h	24 h	4.5	2.0
4	1.0	4 h	24 h	6.0	1.5
5	2.5	4 h	24 h	1.5	0.5
6	5	4 h	24 h	3.5	1.5
CPA	7.5 μg/mL	4 h	24 h	13.0	10.0

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
EMS, CPA: Positive Control (EMS: Ethylmethanesulfonate; CPA: Cyclophosphamide)
200 cells evaluated for each concentration.

Table 13. Summary of Aberration Rates for Experiment IV

Dose Group	Concentration [µL/mL]	Treatment Time	Fixation Interval	mean % aberrant cells	
				incl. Gaps	excl. Gaps
without metabolic activation					
C	0	24 h	24 h	5.0	1.5
S	0	24 h	24 h	5.0	1.0
8	0.50	24 h	24 h	6.0	2.5
9	1.58	24 h	24 h	4.5	2.0
10	5	24 h	24 h	5.5	2.0
EMS	400 µg/mL	24 h	24 h	11.5	10.5
with metabolic activation					
C	0	4 h	24 h	6.5	2.5
S	0	4 h	24 h	4.5	1.5
4	3	4 h	24 h	4.0	2.0
5	4	4 h	24 h	3.5	0.5
6	5	4 h	24 h	4.5	1.5
CPA	7.5 µg/mL	4 h	24 h	11.5	10.5

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
EMS, CPA: Positive Control (EMS: Ethylmethanesulfonate; CPA: Cyclophosphamide)

4.2.1 Clastogenicity. In Experiment III without metabolic activation, the aberration rate of the negative control (1.5 percent) and solvent control (1.5 percent) were within the historical control data of the negative control (0.0 - 4.0 percent, Appendix B). The aberration rates of the concentrations of 0.16 µL/mL (2.0 percent), 0.50 µL/mL (1.0 percent), 1.58 µL/mL (1.0 percent) and 5 µL/mL (1.5 percent) were within the range of the historical control data of the negative control (Appendix B).

In Experiment III with metabolic activation, the aberration rate of the negative control (1.0 percent) and solvent control (2.0 percent) was within the historical control data (0.0 - 4.0 percent, Appendix B). Mean values of 1.5 percent (1.0 µL/mL), 0.5 percent (2.5 µL/mL) and 1.5 percent (5 µL/mL) aberrant cells were calculated for each dose (Table 12). The aberration rate of all dose groups evaluated were within the range of the historical control data.

In Experiment IV without metabolic activation, the aberration rate of the negative control (1.5 percent) and solvent control (1.0 percent) were within the historical control data of the negative control (0.0 - 4.0 percent, Appendix B). The aberration rate of all dose groups evaluated were within the range of the historical control data. Mean values of 2.5 percent (0.5 µL/mL), 2.0 percent (1.58 µL/mL) and 2.0 percent (5 µL/mL) aberrant cells were determined for each dose (Table 13).

In Experiment IV with metabolic activation the aberration rate of the negative control (2.5 percent) and solvent control (1.5 percent) were within the historical control data (0.0 - 4.0 percent, Appendix B). Mean values of 2.0 percent (3 µL/mL), 0.5 percent (4 µL/mL) and 1.5

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percent (5 µL/mL) aberrant cells were found for each dose (Table 13). The aberration rate of all dose groups evaluated were within the range of the historical control data.

4.2.2 Toxicity. Toxic effects of the test item were observed without metabolic activation. In Experiment III, a biologically relevant decrease of the relative mitotic index (decrease below 70 percent relative mitotic index) was noted at doses of 0.50 µL/mL and higher. The highest dose groups evaluated (0.50, 1.58 and 5 µL/mL) induced a decrease of the relative mitotic index down to 68, 51 and 60 percent, respectively (Table 14). In Experiment IV, a biologically relevant decrease of the relative mitotic index (decrease below 70 percent relative mitotic index) was noted at a concentration of 5 µL/mL; the relative mitotic index was 54 percent (Table 15). In Experiment III and IV with metabolic activation, no biologically relevant decrease of the relative mitotic index was noted at the concentrations evaluated (Tables 8 and 11).

Table 14. Experiment III. Number of Polyploid Cells and Mitotic Index: 4 hours Treatment, 24 hours Fixation Period

Dose Group	Concentration [µL/mL]	Polyploid Cells			Mitotic Index Culture			relative [%]
		1	2	Mean	1	2	Mean	
without metabolic activation								
C	0	0	0	0	71	87	79	93
S	0	0	0	0	87	83	85	100
5	0.16	1	0	0.5	86	63	74.5	88
6	0.50	0	0	0	69	46	57.5	68
7	1.58	0	1	0.5	37	49	43	51
8	5	0	0	0	48	54	51	60
EMS	600 µg/mL	0	0	0	50	55	52.5	62
with metabolic activation								
C	0	0	0	0	67	74	70.5	134
S	0	0	0	0	44	61	52.5	100
4	1.0	1	1	1	58	56	57	109
5	2.5	0	0	0	48	57	52.5	100
6	5	0	0	0	75	52	63.5	121
CPA	7.5 µg/mL	0	0	0	40	43	41.5	79

The polyploid cells were determined in 100 cells per culture of each test group.
The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the solvent controls.

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

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Table 15. Experiment IV. Number of Polyploid Cells and Mitotic Index: 4 hours Treatment (with metabolic activation) 24 hours Treatment (without metabolic activation), 24 hours Fixation Period

Dose Group	Concentration [µL/mL]	Polyploid Cells			Mitotic Index Culture			relative [%]
		1	2	Mean	1	2	Mean	
without metabolic activation								
C	0	1	0	0.5	76	87	81.5	130
S	0	0	0	0	62	63	62.5	100
8	0.50	0	1	0.5	54	46	50	80
9	1.58	0	0	0	56	40	48	77
10	5	0	0	0	36	31	33.5	54
EMS	400 µg/mL	0	0	0	46	51	48.5	78
with metabolic activation								
C	0	0	0	0	71	69	70	92
S	0	0	0	0	59	93	76	100
4	3	0	0	0	69	73	71	93
5	4	0	0	0	46	67	56.5	74
6	5	0	0	0	60	57	58.5	77
CPA	7.5 µg/mL	0	0	0	30	39	34.5	45

The polyploid cells were determined in 100 cells per culture of each test group.
The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the solvent controls.

C: Negative Control (Culture Medium)
S: Solvent Control (Ethanol)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

4.2.3 Polyploid Cells. Tables 14 and 15 show the occurrence of polyploid metaphases. No biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item. EMS (400 and 600 µL/mL) and CPA (7.5 µL/mL) were used as positive controls. They showed a distinct and biologically relevant increase of cells with structural chromosome aberrations above our historical control level.

4.2.4 Proliferation Index. The BrdU-technique was used to detect a possible cell cycle delay after treatment with the test item. In Experiment III, the values of the proliferation index of the negative controls were 1.37 (without metabolic activation) and 1.33 (with metabolic activation) (Table 16). The proliferation index of the highest dose groups evaluated was 1.35 (without metabolic activation) and 1.32 (with metabolic activation) at a concentration of 5 µL/mL. In Experiment IV, the values of the proliferation index of the negative controls were 1.41 (without metabolic activation) and 1.21 (with metabolic activation) (Table 17). The proliferation index of the highest dose groups evaluated without metabolic activation (5 µL/mL) was 1.40. The proliferation index of the highest dose group evaluated with metabolic activation (5 µL/mL) was 1.27. There was no biologically relevant decrease of the proliferation index.

Table 16. Experiment III. Proliferation Index Determined by BrdU-Labeling

Dose Group	Concentration [µL/mL]	Treatment Time	Proliferation Index	OT 1			OT 2		
				1. Mitosis	2. Mitosis	3. Mitosis	1. Mitosis	2. Mitosis	3. Mitosis
without metabolic activation									
S	0	4 h	1.37	68	32	0	58	42	0
8	5	4 h	1.35	61	43	0	71	29	0
with metabolic activation									
S	0	4 h	1.33	72	29	0	62	38	0
6	5	4 h	1.32	68	32	0	68	32	0

S: Solvent Control

Table 17. Experiment IV. Proliferation Index Determined by BrdU-Labeling

Dose Group	Concentration [µL/mL]	Treatment Time	Proliferation Index	OT 1			OT 2		
				1. Mitosis	2. Mitosis	3. Mitosis	1. Mitosis	2. Mitosis	3. Mitosis
without metabolic activation									
S	0	24 h	1.41	60	40	0	59	41	0
10	5	24 h	1.40	65	35	0	56	44	0
with metabolic activation									
S	0	4 h	1.21	84	16	0	74	26	0
6	5	4 h	1.27	80	31	0	73	27	0

S: Solvent Control

5.0 CONCLUSION

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, F-T jet fuel did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used. Therefore, F-T jet fuel is considered to be non-mutagenic in this bacterial reverse mutation assay.

During the described *in vitro* chromosomal aberration test and under the experimental conditions reported, the test item F-T jet fuel did not induce structural chromosomal aberrations in human lymphocyte cells. Therefore, F-T jet fuel is considered to be non-clastogenic in this chromosome aberration test.

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APPENDIX A. HISTORICAL LABORATORY CONTROL DATA FOR REVERSE MUTATION ASSAY

Table A-1. Historical laboratory control data of the negative control (in 2004 - 2006) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	28.6	109.2	17.2	12.5	269.7
SD	6.0	14.6	4.9	4.3	55.5
Min	18.0	75.0	6.0	5.0	166.0
Max	54.0	171.0	30.0	31.0	394.0
RSD [%]	21.0	13.4	28.5	34.1	20.6
n =	533	533	526	514	427

S9:   metabolic activation
Mean: mean of revertants/plate
Min.:   minimum of revertants/plate
Max.:   maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table A-2. Historical laboratory control data of the positive control (in 2004 - 2006) without S9 (-S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	821.4	974.9	1022.1	183.7	2078.3
SD	329.5	227.4	244.9	60.0	381.9
Min	271.0	235.0	89.0	94.0	670.0
Max	2420.0	2235.0	1630.0	1132.0	3357.0
RSD [%]	40.1	23.3	24.0	32.7	18.4
n =	533	532	528	514	429

S9:   metabolic activation
Mean: mean of revertants/plate
Min.:   minimum of revertants/plate
Max.:   maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

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Table A-3. Historical laboratory control data of the negative control (in 2004 - 2006) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	39.4	116.5	13.1	13.3	302.2
SD	7.7	14.8	3.6	4.6	71.1
Min	16.0	83.0	6.0	6.0	153.0
Max	71.0	168.0	31.0	36.0	594.0
RSD [%]	19.6	12.7	27.3	34.5	23.5
n =	532	535	526	522	428

S9:   metabolic activation
Mean: mean of revertants/plate
Min.:   minimum of revertants/plate
Max.:   maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

Table A-4. Historical laboratory control data of the positive control (in 2004 - 2006) with S9 (+S9)

	TA 98	TA 100	TA 1535	TA 1537	TA 102
Mean	1832.0	1851.2	134.2	227.1	837.3
SD	815.5	691.6	72.8	146.2	284.4
Min	121.0	298.0	38.0	41.0	358.0
Max	3430.0	3366.0	1090.0	2289.0	2018.0
RSD [%]	44.5	37.4	54.3	64.4	34.0
n =	532	536	526	522	427

S9:   metabolic activation
Mean: mean of revertants/plate
Min.:   minimum of revertants/plate
Max.:   maximum of revertants/plate
SD: Standard Deviation
RSD: Relative Standard Deviation
n: Number of control values

APPENDIX B. HISTORICAL LABORATORY CONTROL DATA FOR CHROMOSOMAL ABERRATION ASSAY

Table B-1. Historical laboratory control data of the negative control (2000 - 2006)

	NC Number of aberrant cells			
	(-S9)		(+S9)	
	+Gaps	-Gaps	+Gaps	-Gaps
mean [%]	3.0	1.7	1.7	1.0
SD [%]	1.17	1.10	1.68	1.18
RSD [%]	39.4	63.7	98.9	113.8
min [%]	1	0	0	0
max [%]	5	4	5.5	4
n	26	26	15	15

NC:	Negative Control
mean:	mean number of aberrant cells
SD:	Standard Deviation
RSD:	relative Standard Deviation
min.:	minimum number of aberrant cells
max.:	maximum number of aberrant cells
n:	Number of assays

Table B-2. Historical laboratory control data of the positive control (2000 - 2006)

	PC Number of aberrant cells			
	(-S9)		(+S9)	
	+Gaps	-Gaps	+Gaps	-Gaps
mean [%]	20.2	18.5	18.0	15.9
SD [%]	7.21	7.55	6.99	6.62
RSD [%]	35.7	40.8	38.9	41.8
min [%]	10	9	8.7	8
max [%]	40.7	40.7	33	30
n	26	26	14	14

PC: Positive Controls (+S9 CPA, -S9 EMS)
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.:   minimum number of aberrant cells
max.:   maximum number of aberrant cells
n: Number of assays

LIST OF ABBREVIATIONS

bio	biotin mutation
BrdU	bromodeoxyuridine
CA	chromosome aberration
chI	nitrate reductase mutation
CPA	cyclophosphamide
DMSO	dimethyl sulfoxide
EMS	ethylmethanesulfonate
F-T	Fischer Tropsch
his	histidine
OECD	Organisation for Economic Co-operation and Development
PHA	phytohemagglutinin
PI	proliferation index
rfa	deep rough mutation